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Targeted gene transfer into hepatoma cells with lipopolyamine-condensed DNA particles presenting galactose ligands: A stage toward artificial viruses

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ABSTRACT **Optimal *in vitro* gene delivery with cationic lipids requires an excess of cationic charges with respect to DNA phosphates. In these conditions, *in vivo* delivery will be hampered by interference from cationic lipid-binding macromolecules either circulating or in the extracellular matrix. To overcome this problem, we are developing a modular transfection system based on lipid-coated DNA particles reminiscent of enveloped viruses. The particle core consists of the lipopolyamine-condensed nucleic acid in an electrically neutral ratio to which other synthetic lipids with key viral properties are hydrophobically adsorbed. As a first result, we have found that a good transfection level can be achieved simply with the neutral core particle, provided a zwitterionic lipid (dioleoyl phosphatidylethanolamine) is added to completely coat the DNA. Addition of lipids bearing a fusogenic or a nuclear localization peptide head group to the particles does not significantly improve an already efficient system, in contrast to polylysine-based gene transfer methods that rely on lysosomotropic or fusogenic agents to be effective. This emphasizes the distinctive properties of the lipopolyamines, including cell membrane destabilization, endosome buffering capacity, and possibly nuclear tropism. Most importantly, addition of lipids with a triantennary galactosyl residue drives the neutral nucleolipidic particles to the asialoglycoprotein receptor of human hepatoma HepG2 cells: Transfection increases ≈1000-fold with 25% galactolipid. This receptor-mediated process is saturable and slightly less efficient than receptor-independent transfection obtained *in vitro* with a large excess of cationic lipid alone. Yet, electrically silent particles may provide an attractive solution for gene transfer *in vivo* where their external saccharide coat should allow them to diffuse within the organism and reach their target cells.**

The prospect of curing inherited and acquired diseases through gene therapy has engendered considerable effort toward the development of gene transfer vectors (1–3). Most ongoing human gene therapy protocols rely on recombinant retroviral and adenoviral vehicles, which, besides their limited carrier capacity, risk encountering acute safety and immunological problems with large scale or repeated use. Synthetic vectors, although currently orders of magnitude less efficient than biological vectors, are increasingly being considered to be possible solutions as well (4–7).

Synthetic gene transfer molecules are designed to form multimolecular aggregates with plasmid DNA and to bind the resulting particles to the cell surface in such a way as to trigger endocytosis and endosomal membrane disruption. Broadly speaking, two classes of vectors have been developed so far.

(*i*) Polymeric DNA-binding cations (such as polylysine, protamine, and cationized albumin) linked to cell targeting ligands [such as asialoorosomucoid (8), insulin (9), galactose (10), or lactose (11)] trigger receptor-mediated endocytosis into hepatocytes or into a wider spectrum of cells when linked to transferrin (12). The transgene is thought to be carried to the acidic endosomal compartment however, where it stays or is extensively degraded unless lysosomotropic agents are present. Nonetheless, very efficient gene transfer has been recently achieved with a hemisynthetic virus resulting from the coupling of DNA/polylysine-transferrin particles to a defective adenovirus (13) or to a fusogenic peptide (14).

(*ii*) Cationic amphiphiles provide receptor-independent gene transfer into various cell types, as the cationized DNA binds to anionic residues on the cell surface. Many compounds bearing essentially a single tertiary (15) or quaternary ammonium head group (16–23) have been tested *in vitro*. Efforts in this laboratory have been directed to the use of natural protonable polyamines as lipid head groups (24), which provide additional properties, such as buffering capacity and DNA condensation. Accordingly, although drawing-up of a hit parade of transfection vehicles seems a delicate task, lipopolyamines have been shown to be consistently at least as efficient as the other types of cationic lipids in transfecting various cell types *in vitro* (7, 25). More recently, amphipathic cationic polypeptide/lipid mixtures and dendrimeric polyamines have been shown to carry genes into many cell types as well (26, 27).

In vivo gene transfer is a much more difficult task to achieve with nonviral methods. *In vitro* transfection with cationic lipids is best obtained when the nucleolipidic particles bear a strong net positive charge (7, 24). Yet a cationic lipid-coated particle has little chance of reaching its target *in vivo* because it will be either coated with serum proteins (such as heparin, albumin, and lipoproteins) or bind to the anionic glycosaminoglycan extracellular matrix. Some success has nevertheless been obtained (28–35), often by using amounts of cationic lipids that are suboptimal for completely neutralizing the DNA.

Here we describe artificial viral-like particles that in principle should avoid the aforementioned difficulties. These gene transfer vehicles are based on electrically neutral lipopolyamine-condensed DNA particles that should display a decreased nonspecific binding and to which other lipidic components can be added to mimic key viral functions. Thus upon mixing several synthetic lipids (including spermine, glycoside, fusogenic, and nuclear localization head groups) with plasmid DNA, the spermine residues should spontaneously compact the nucleic acid into lipid-coated particles, leaving at their surface molecular signals for cell targeting, entry into the cytoplasm, and trafficking to the nucleus. Cell-specific interaction was indeed obtained by including multiantennary galactosyl ligands in the transfecting particles that recognize the Gal/GalNAc receptors present on certain cell types, such as

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Abbreviations: DOPE, dioleoylphosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; MPB, maleimidophenylbutyrate; NLS, nuclear localization signal; SV40, simian virus 40; N-t-HA, N-terminal peptide of hemagglutinin; RLU, relative light unit.

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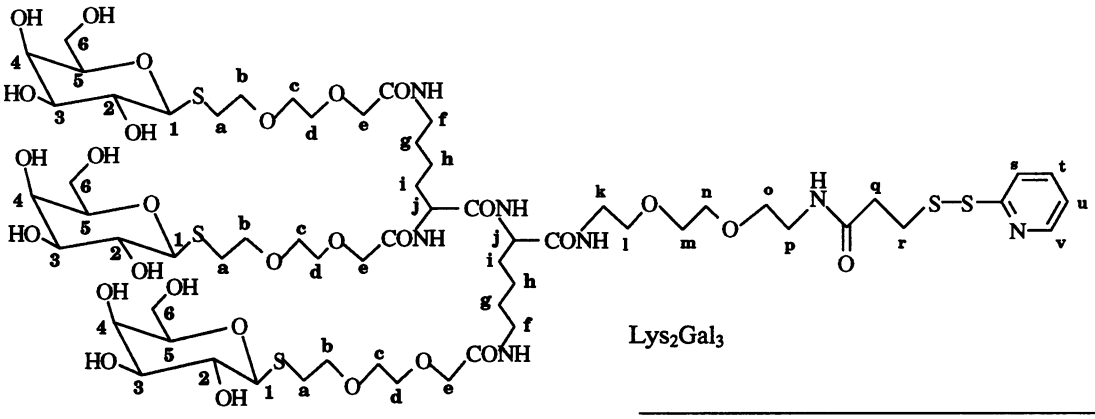
hepatocytes, macrophages, and metastases, and that trigger receptor-mediated endocytosis. Although the remainder of our initial goal was not fully reached, the modular transfection system described here should enable the properties of lipopolyamines to be extended to gene transfer *in vivo*.

MATERIALS AND METHODS

Addition of Functional Groups to Lipids. The terminal amine groups of dipalmitoylphosphatidylethanolamine (DPPE) and glycyldioctadecylamide were coupled to functional thiol-containing residues via the activated maleimido ester *N*-succinimidyl-(*p*-maleimidophenyl)butyrate (succinimidyl-MPB, Pierce). The thiol-protected galactoside head group Lys_2Gal_3



Lys_2Gal_3

was obtained by coupling (ethyldimethylaminopropylcarbodiimide) the terminal lysine carboxyl function of the triantennary dipeptide with a heterobifunctional spacer obtained in turn by coupling ethyldimethylaminopropylcarbodiimide 1,8-diamino-3,6-dioxaoctane with 2-carboxyethyl 2-pyridyl disulfide. ^{1}HNMR (^2H_2O): 1.39–1.93 [m, 12H, 2 × (2H-g, 2H-h, and 2H-i)], 2.7 (t, 2H-q, J = 6.4 Hz), 2.9–3.13 (m, 8H, 2H-r, and 3 × 2H-a), 3.16–3.49 (m, 8H, 2H-k, 2H-p, and 2 × 2H-f), 3.54–3.84 [m, 41H, 3 × (H-2, H-3, H-5, H-6a, H-6b, 2H-b, 2H-c, and 2H-d), 2H-l, 2H-m, 2H-n, and 2H-o], 4 (d, 3H, 3 × H-4), 4.08 (s, 4H, 2 × 2H-e), 4.16 (s, 1 × 2H-e), 4.28 (m, 1H, 1 × H-j), 4.39 (m, 1H, 1 × H-j), 4.53 (d, 3H, 3 × H-1, J = 9 Hz), 7.34 (m, H-s), 7.87 (m, 2H, H-t, and H-u), and 8.84 (m, H-v). MS (ES+) calculated for $C_{62}H_{107}N_7O_{29}S_5$ = 1573.57. Found m/z = 807.5 ($M+H^+ + K^+$); 799.44 ($M+H^+ + Na^+$); 788.45 ($M+2H^+$).

The triantennary galactolipid DPPE-MPB-Lys_2Gal_3 was synthesized by first deprotecting Lys_2Gal_3 (0.1 mM) with tris-(2-carboxyethyl) phosphine (Pierce, 1.1 equivalents) in 10 mM sodium phosphate (pH 5.5), followed by reaction with DPPE-MPB, which was prepared as described (36). The desired amount of DPPE-MPB for a transfection triplicate was bath-sonicated in 40 μl of a 1:10 dilution of Hepes-buffered saline (HBS), pH 6.7/ethanol, 1:1 (vol/vol), and was coupled to the thiotrigalactoside (20 min at room temperature, followed by TLC). The desired amount of Transfectam was then added, the solution was vortex-mixed, and 6 μg of plasmid was added. After vortex mixing, 2.9 ml of Dulbecco's modified Eagle's medium was added and the transfection mixture was distributed among the wells. Alternatively, the coupling reaction was performed after the addition of Transfectam or after the formation of the cationic lipid/DNA particles in ethanol-free solution, with very similar results. Glycine dioctadecylamide (24) was coupled quantitatively to *N*-succinimidyl-(*N*-maleimidophenyl)butyrate in dichloromethane and the compound was redissolved in 40 mM ethanol. The reactive lipid (25 μl) was added to 1 ml of 1 mM nuclear localization signal (NLS)-Cys peptide Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro-Tyr (Neosystem, Strasbourg, France) in 5 mM Tris·HCl (pH 8) in 9:1 ethanol/water. After completion of the reaction (monitored with Ellmann's reagent), the desired lipopeptide was precipitated (−80°C) with 80% yield as determined by UV. The N-terminal peptide of hemagglutinin (N-t-HA)-Cys peptide Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Asn-Gly-Trp-Glu-Gly-Met-Ile-Asp-Gly was coupled after essentially the same procedure, in 1:1 ethanol/water.

Preparation of the Lipid–DNA Complexes and Cell Transfection. The lipopolyamine (24), 2 or 20 mM in ethanol (Transfectam, Promega), was mixed with pGL2-Luc (Promega), pCMV-Luc (O. Feugeas, Strasbourg Hospital, France), or pCMV-β-Gal (B. Demeneix, Museum, Paris) in 150 mM NaCl (37). Plasmids were purified by Qiagen column chromatography. The lipopolyamine/dioleoylphosphatidylethanolamine (DOPE)/plasmid complexes were formed by mixing the desired amounts of lipopolyamine and DOPE (Sigma; 20 mM in ethanol containing a trace of chloroform) prior to dilution with 150 mM NaCl. The formation of particles bearing galactosyl residues is described above.

Swiss 3T3 murine fibroblast (J. P. Beck, Faculte de Pharmacie, Strasbourg, France) and human hepatoma HepG2 (American Type Culture Collection) cell lines were cultured and seeded at 50,000 cells per well in 24-well dishes (Falcon) as described (37, 38). After 12 h, cells were rinsed and the transfection mixture was added. Fetal calf serum [10% (vol/vol)] was added after 4 h (Fig. 1) or 5 h (Figs. 2 and 3), and transgene expression was monitored 24 h later. β-Galactosidase reporter gene expression was detected histochemically as described (39). Luciferase activity was quantitated by photon counting (Biolumat LB 9500; Berthold, Nashua, NH) with a commercial kit (Promega). All transfection experiments were done in triplicate and are expressed as the mean ± SD.

RESULTS

Transfection with the Core Particle Alone. *In vitro* gene transfer with cationic amphiphiles is most efficient when the nucleolipidic particles bear a strong net positive charge (7, 24). Transfection also depends to a large extent on the conditions of formation of the DNA–lipid complexes (37). We have optimized conditions for lipopolyamine-mediated introduction of pCMV-Luc and pCMV-β-Gal reporter plasmids into NIH 3T3 and HepG2 cells (Fig. 1). Luciferase activity increased by more than two orders of magnitude for both cell lines when the cationic lipid/anionic DNA charge ratio was increased from 1.5 to 6. This technique is very efficient, since reporter gene expression values of up to 2×10^{10} and 1×10^8 relative light units (RLU)/mg of protein could be obtained with 5 μg of DNA for 3T3 and HepG2 cells, respectively. By

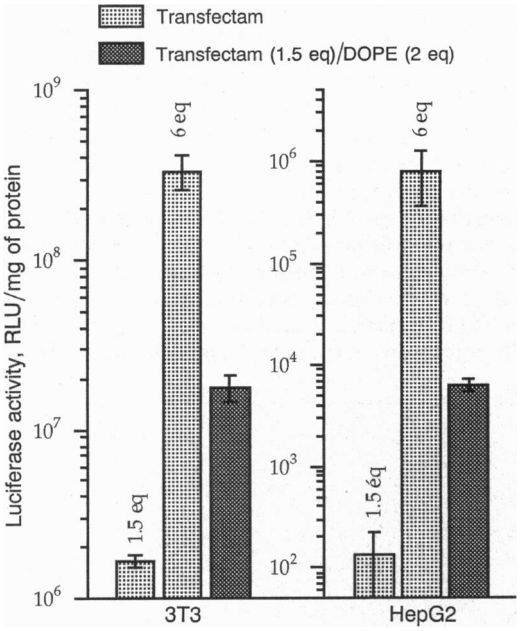


FIG. 1. Zwitterionic lipid DOPE restores in part the efficiency drop observed upon decreasing the cationic lipid/DNA ratio from 6 to 1.5. Expression of the pCMV-Luc reporter gene (1 μg in fibroblast and 2 μg in hepatoma cell lines) was monitored after transfection mediated by the indicated amounts of lipopolyamine or lipopolyamine/DOPE mixtures. The cationic lipid/DNA charge ratio was calculated by assuming that 1.25 μg of cationic lipid and 1 μg of DNA correspond to 3 nmol of cationic and anionic charges, respectively. The amount of DOPE is expressed as mol equivalents with respect to the cationic lipid.

using the same experimental conditions, β-galactosidase expression was revealed histochemically in 55% (±5%; mean ± SD of three random fields) of the 3T3 cells.

In vivo gene transfer using particles with a high cationic charge density may be perturbated after opsonization with anionic circulating proteins. Electrostatic binding of the particles to the strongly anionic tissue matrix is expected to occur as well, which would confine them to the injection site. On the other hand, nearly neutral particles are comparatively inefficient (Fig. 1). Lipopolyamines bear three to four cationic charges per molecule (7), and a neutral lipopolyamine–DNA complex contains only an average of one lipid molecule for every three or four nucleic bases; the plasmid probably lacks enough lipid molecules to be properly coated, which explains the low efficiency observed. To test this hypothesis, a zwitterionic lipid was added to the complex, to fill in the coat without increasing the overall cationic charge of the particle. DOPE was chosen because it has putative membrane-destabilizing properties that enhance transfection with other molecules (16, 18, 26). Indeed, for both cell lines, half of the efficiency drop observed upon decreasing the charge ratio to 1.5 could be recovered with the addition of 2 mol of DOPE per mol of cationic lipid (Fig. 1). To find the best conditions compatible with *in vivo* transfection, β-galactosidase reporter gene expression was measured for various DOPE/Transfectam combinations. Fig. 2 shows that the addition of 2 mol of DOPE per mol of lipopolyamine leads to half as many positively stained cells as the optimal conditions carried out with a large excess of lipopolyamine alone.

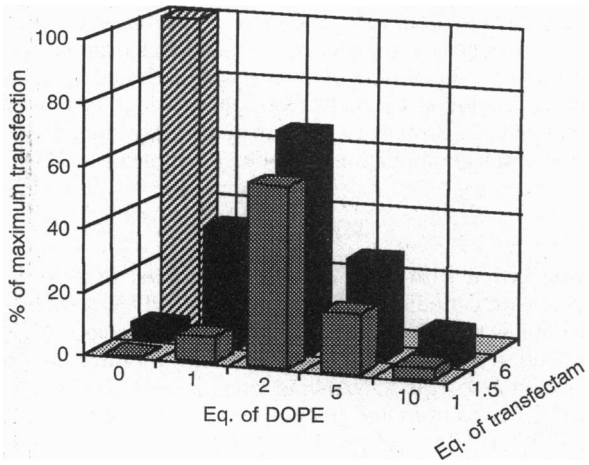


FIG. 2. Electrically neutral particles gain the maximum efficiency after addition of 2 mol equivalents (Eq.) of the zwitterionic lipid DOPE. 3T3 cells were transfected with 2 μg of pCMV-β-Gal and various combinations of lipopolyamine and DOPE (see Fig. 1). Enzyme activity was revealed by using 5-bromo-4-chloro-3-indolyl β-D-galactoside and the percentage of blue cells (mean of three random fields) was determined.

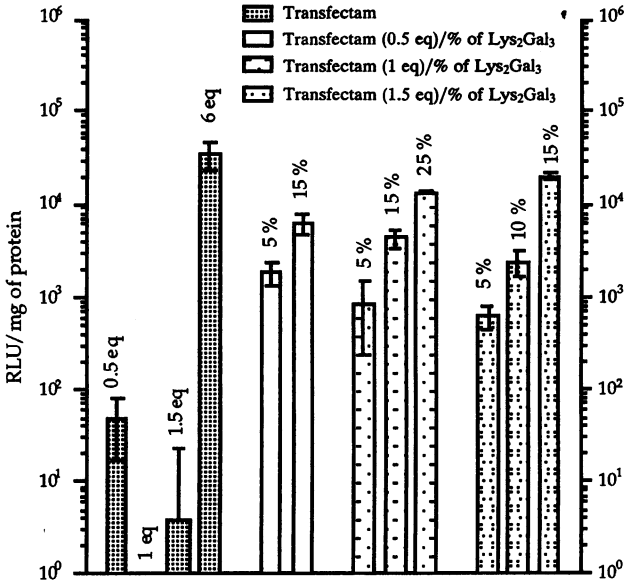


FIG. 3. Targeting of the asialoglycoprotein receptor of hepatoma cells with neutral lipopolyamine-condensed DNA particles bearing galactose residues. HepG2 cells were transfected with 2 μg of pGL2-Luc and various lipids (0.5–6 cationic lipid/DNA ionic ratio and 5–25 mol % of triantennary galactolipid; see Fig. 1 for a definition of ionic ratio). Transfection efficiency of the neutral particles, as measured by the luciferase activity after 24 h, was increased ≈1000-fold in the presence of the galactolipid.

Addition of Lipopeptides with Fusogenic or Karyophilic Properties to the Core Particle. Viruses have evolved efficient solutions for crossing the plasma and nuclear membrane barriers, such as fusogenic and karyophilic proteins. To develop a modular transfection system based on lipid components expressing key viral functions through their head groups, we have synthesized two lipopeptides. The glycyldioctadecylamide lipid moiety of Transfectam was stepwise linked to C-terminal Cys-bearing peptides, N-t-HA and simian virus (SV40)-NLS, through a heterobifunctional spacer (*N*-succinimidyl-(*p*-maleimidophenyl)butyrate). N-t-HA includes the 20 N-terminal amino acids of the influenza virus hemagglutinin that have been shown to form an α-helix and to display membrane disrupting properties when the Asp and Glu residues are protonated (40, 41). SV40-NLS includes the undecapeptide NLS of the SV40 large tumor antigen that is able to promote protein import into the nucleus (42, 43). However, in admixture with lipopolyamines, none of these lipopeptides

significantly enhanced the transfection of 3T3 or HepG2 cells (data not shown; see also next paragraph).

In a different context, covalent attachment of N-t-HA to polylysine was shown to result in the increase (several orders of magnitude) of transferrin receptor-mediated gene transfer (14). However, transferrin-polylysine–DNA complexes alone are extensively degraded in the lysosomes, and this vehicle is rather inefficient unless a weak base such as chloroquine is present in the medium (12, 44), which explains the enhancement observed in the presence of a fusogenic peptide. A similar behavior was noticed for other polylysine-based vectors (10, 11). Lipopolyamines do not require a receptor for cell binding, and the coated DNA may reach the cytoplasm after membrane disruption along the endosomal route, because of the nonbilayer forming tendency of the lipid (7). On the other hand, the spermine head is not fully protonated ($pK_4 = 5.5$) under physiological conditions, which leaves it with a substantial endosome buffering capacity (7, 25). These constitutive properties could explain why N-t-HA (as well as chloroquine, data not shown) cannot further enhance an already efficient lipopolyamine-mediated gene transfer process.

The SV40-NLS peptide contains a polycationic stretch that may compete with spermine for binding to DNA and disappear from the particle surface. However, lipopolyamine-coated particles should also have an intrinsic nuclear tropism. Indeed, whatever their actual mechanism of entry into the nucleus, the distribution between the cytoplasmic and nuclear compartments will favor accumulation in the latter one since the cationic particles bind to chromatin. There, plasmid uncoating may simply be the result of a competitive exchange of the cationic lipid between DNA from the exogeneous source and that from a large pool of chromatin.

Targeting of Hepatoma Cells with Galactose-Bearing Lipids. Because addition of viral functions such as membrane disruption and nuclear tropism to lipopolyamines was not effective, we tried to substitute the rather nonspecific binding of positively charged particles to cells with a more specific interaction. The Gal/GalNAc receptor present at the surface of mammalian hepatocytes (i.e., the asialoglycoprotein receptor) has been well characterized (45) and was used for the targeting, *in vitro* and *in vivo*, of polylysine-based gene delivery systems (for review, see ref. 46). The efficiency of these targeting systems depends critically on the presence of lysosomotropic agents or membrane-disrupting peptides (as discussed above) and on DNA compaction (47). Since lipopolyamines seem to constitutively possess these properties, we studied the possibility of transfecting human hepatoma HepG2 cells with neutral lipopolyamine-condensed DNA particles carrying galactose residues. Oligomeric structures are required for efficient binding to the galactose receptor (48). Because of the structural flexibility offered by the synthetic approach (49), several triantennary galactolipids were synthesized that gave essentially the same results. Targeted transfection of hepatoma cells is shown in Fig. 3. The control experiments with low-charge particles (0.5–1.5 equivalents) lacking the galactolipid gave scattered values close to baseline. Addition of the galactolipid up to 25 mol % increased transfection efficiency >1000-fold, approaching the value obtained with a large excess (6 equivalents) of Transfectam alone (yet through a different mechanism). Higher amounts of the glycolipid led to a plateau. To demonstrate that the enhancement observed was indeed from recognition of the neutral particles by the galactose receptor of HepG2 cells, we have performed several control experiments. (*i*) The preformed particles containing DPPE-MPB were first reacted with 2-mercaptoethanol (3 equivalents) or Cys (3 equivalents) to derivatize the maleimido functions. Subsequent addition of the triantennary Lys_2Gal_3 thiol head, which could no longer be conjugated to the particles, did not permit recovery of transfection capacity beyond the control level; in the reverse order of addition, however, the targeting effect was observed. (*ii*) Addition of asialofetuin (up to 0.6 mg per well), a ligand of the hepatocyte galactose receptor, was unable to inhibit transfection ($3 \pm 1 \times 10^6$ RLU). This might be surprising; however, because of multiple binding sites, the particles could engage in an interaction of very high affinity that is difficult to displace by asialofetuin. A similar effect was observed for mannosylated liposomes (50). (*iii*) Experiments similar to the ones described in Fig. 3 were performed with 3T3 fibroblasts, which lack the galactose receptor at their surface. Interestingly, transfection efficiency decreased markedly (100-fold with 30 mol % of Lys_2Gal_3), showing not only that receptor-mediated transfection is absent but also that here the galactosyl coat interferes with ionic binding of the particles to the cell surface [this "stealth" effect was also observed with an oligoethylene glycol-bearing lipid (J.S.R., unpublished results)]. (*iv*) Finally, addition of chloroquine (0.1 mM, 2 h) or addition of the fusogenic N-t-HA peptide, either free (10 μM) or conjugated to the preformed targeting particles through a disulfide bridge (20 mol %), did not increase transfection. Replacement of the lipopolyamine with the permanent charge-bearing lipid DOTMA (16) resulted in a complete loss of the targeting effect brought about by Lys_2Gal_3. These results reinforce the hypothesis that lipopolyamines possess intrinsic buffering and membrane-disrupting properties that are of crucial importance subsequent to the cell surface binding step. Thus, the experiments clearly demonstrate that receptor-mediated endocytosis of DNA can be achieved with mixed lipid particles presenting a specific ligand.

DISCUSSION

Nonviral vectors are safe and in principle can carry genomic DNA into eukaryotic cells (51). Unfortunately, the low *in vivo* efficiency presently precludes their use for gene therapeutical purposes. *In vitro*, however, several such cationic compounds intrinsically display a very high efficiency (i.e., $>10^8$ light units per mg of cell protein or per 10^6 cells, for transfection of cell lines such as 3T3, COS, or CV-1 with a strong promoter-driven luciferase plasmid). Besides the lipopolyamines, these include dendrimeric (27) and other branched (O. Boussif and J.-P.B., unpublished results) polyamines; efforts to further improve their performances via externally added or covalently linked residues aimed at receptor binding or membrane disrupting

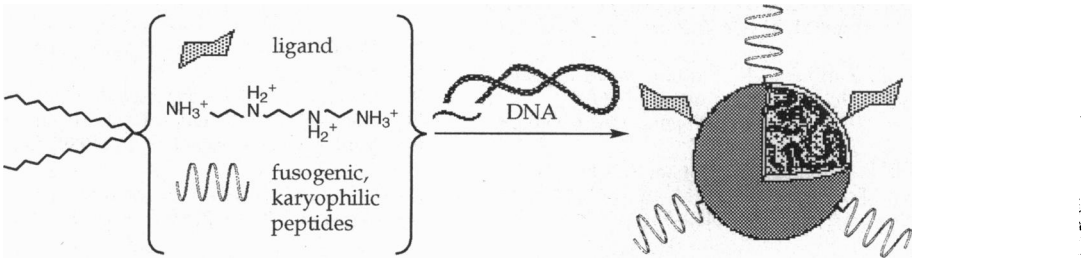


FIG. 4. Self-assembly of DNA and lipids bearing various functional head groups that collapse into an artificial gene delivery vehicle.

have so far failed. Under suboptimal conditions (i.e., neutral transfecting particles), however, such additional functions may play a crucial role, as shown (27) and in the present study. In contrast to this, comparatively unefficient vectors such as polylysine conjugated to cell-binding ligands gain much efficacy in the presence of membrane-disrupting peptides (10, 11, 14, 44); in particular, coupling to defective adenoviral particles leads to luciferase expression levels even higher than those found above (13, 44). These divergent behaviors probably reflect, among others, the fate of DNA after receptor-independent cell binding via ionic forces or receptor-mediated endocytosis. The performances obtained with all these optimal delivery systems on primary cells would often be sufficient to envisage a therapeutical use.

The situation is different *in vivo*, where artificial vectors remain far behind the natural ones; yet their favorable "inertness" justifies further exploration of nonviral alternatives. Since *in vivo* diffusion and gene transfer appear to be incompatible with particles of high cationic charge density, we optimized conditions where the cationic lipid and anionic DNA charges nearly compensate each other: Filling the particles with DOPE greatly increased their efficiency, and indeed promising results have recently been obtained *in vivo* (B. Demeneix, D. Scherman, and B. Schwartz, personal communication). Along similar lines, a second approach to gene delivery that is shown here mimics some of the useful properties of viruses. To avoid cationic particles, the role of lipopolyamine here is restricted to condensing DNA and to providing a hydrophobic core to bind other lipids expressing viral functions. Such a modular gene delivery vehicle (Fig. 4) could easily be adapted to various cellular contexts by adjusting the nature and ratio of its components. Results obtained so far highlight only the compacting and cell targeting functions of these artificial viruses. However, the challenge represented by gene therapy certainly will stimulate chemists and molecular biologists to develop more efficient solutions to artificial gene delivery and to develop still safer viral vectors.

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